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# Genetic Diversity of *Rehmannia glutinosa* Genotypes Assessed by Molecular Markers

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Random amplified polymorphic DNA (RAPD) markers were used to identify the genetic diversities among and within varieties and landraces of *Rehmannia glutinosa*. Polymorphic and reproducible bands were produced by 10 primers out of total 20 primers used in the experiment. In RAPD analysis of the 11 genotypes, 64 fragments out of 73 amplified genomic DNA fragments were polymorphic which represented an average 6.4 polymorphic fragments per primer. Number of amplified fragments with random primers ranged from 2 (OPA-1) to 13 (OPA-11) and varied in size from 200 bp to 1,400 bp. Especially, OPA-10, OPA-11 and OPA-19 primers showed specific bands for varieties of Korea Jiwhang and Jiwhang il ho, which could be useful for discriminating from other varieties and landraces of *R. glutinosa*. Percentage polymorphism ranged from a minimum of 50% (OPA-1) to a maximum of 100% (OPA-11), with an average of 87.7%. Similarity coefficients were higher in the genotypes of Korea Jiwhang and Jiwhang il ho than in other populations. In cluster analysis, genotypes of Korea Jiwhang, Jiwhang il ho, and Japanese accession were separated from those of other varieties and landraces. Average of genetic diversity within the population ($H_S$) was 0.110, while average of total genetic diversity ($H_T$) was 0.229. Across all RAPD makers the $G_{ST}$ value was 0.517, indicating that about 52% of the total genetic variation could be explained by RAPDs differences while the remaining 48% might be attributable to differences among samples. Consequently, RAPD analysis was useful method to discriminate different populations such as domestic varieties and other landraces. The results of the present study will be used to understand the population and evolutionary genetics of *R. gllutinosa*.

**Key words** : *Rehmannia glutinosa*, randomly amplified polymorphic DNAs, identification, genetic diversity

## Introduction

*Rehmannia glutinosa* Liboshitz is a member of the Scrophulariaceae family whose roots are used in Chinese medicine for anemia, hemoptysis, and gynecological diseases. A variety of compounds have been isolated from root extracts; catalpol, with diuretic activity, is one of the main ingredients [15]. However, little is known about the chemical composition of leaves because they are not used medicinally.

According to clinical studies, these herbs, which have been added to the list of useful items over the centuries, can greatly increase the efficacy of modern drug treatment, and reduce their side-effects. There are three forms of the herb, called Di Huang in China, that are currently used Saeng Jiwhang (shengdihuang: Chinese), Suk Jiwhang (shouduhuang: Chinese) and dried Jiwhang. Sheng Jiwhang or fresh root is used to reduce inflammation and is included in many formulas for autoimmune disorders. Suk Jiwhang and Dried Jiwhang are used as a nourishing tonic and subsequent dehydration [2].

The Korean botanical name "Jiwhang" is involved in the genus *Rehmannia* of the family Scrophulariaceae. In Korean and Chinese pharmacopoeia, Jiwhang is currently defined as the root of either *R. glutinosa* or related species belong to same genus [6]. In Japanese pharmacopoeia, however, both *R. glutinosa* and *R. glutinosa* var. *purpurea* Makino are defined as Jiwhang. Although *R. glutinosa*'s varieties including Korea Jiwhang and Jiwhang il ho are developed within a country, genetic improvement is limited by a poor

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knowledge of genetic diversity within the species.

The evaluation of genetic diversity and construction of linkage maps would promote the efficient use of genetic variations in the breeding program [11]. DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers [13]. Polymerase chain reaction (PCR) based approaches developed in recent years such as random amplified polymorphic DNA [18], inter-simple sequence repeat [21], amplified fragment length polymorphism [17] and microsatellites [19] have extensively been used for investigating genetic relatedness and diversity in plant populations and cultivars [1,16].

Especially, RAPD technique is popular among the various DNA marker assisted techniques currently available [18], because of its speed, low cost, and necessity of only minute amounts of plant material. In spite of its dominant marker nature, RAPD can be used to assess fixation indexes and population genetic parameters when appropriate statistical analysis is used [5,8,14].

So far only a few attempts have been made to characterize the genetic diversity in *R. glutinosa* by molecular markers. The aims of this study are (1) to access the genetic diversity and relationships among three varieties and eight landraces and (2) to investigate the genetic variation within varieties and landraces in *R. glutinosa* at DNA level.

## Materials and Methods

### Plant materials and DNA extraction

A total of 11 populations including three varieties and eight landraces were evaluated in this study. Domestic varieties, Jiwhang il ho, Korea Jiwhang and Daekyeong Jiwhang, were collected from National Institute of Crop Science, Suwon, Kyonggi Province, Korea. The domestic landraces of *R. glutinosa* were from seven regions including Euisung in Korea and the imported one were from Japan (Table 1).

The plant samples were frozen in liquid nitrogen and ground in a mortar to become a fine powder. DNA was extracted from young leaves using the method described by Dellaporta (1983) [4]. The relative concentration of extracted DNA was estimated with the help of Nano Drop ND-1000 (Dupont Agricultural Genomics Laboratory, America), and the final DNA concentration was adjusted to 10 ng/μl.

### RAPD analysis

The RAPD analysis was carried out using the following mixture: 2.0 μl genomic DNA (10 ng/μl), 2.0 μl primer (5 μM), 2.0 μl dNTPs (250 μM total), 0.2 μl e-*Taq*-polymerase (5 U/μl), 2.5 μl 10× buffer containing 25 mM $MgCl_2$, 16.3 μl distilled water, for a total of 25 μl reaction mixture. The e-*Taq*-polymerase and buffer were purchased from SOLGENT Inc. (KOREA).

Because RAPD-PCR is sensitive to reaction parameters, 20 primers supplied by OPERON Technologies Inc. (Alameda, CA) were initially screened against four populations selected from varieties and landrace of *R. glutinosa*. The effects of template DNA and random primer concentrations, *Taq* polymerase concentration, and different times and temperatures during the annealing stage of PCR amplification were examined. To establish the optimum annealing temperature, ten annealing temperatures ranging

Table 1. Information of *R. glutinosa* used in study

| POP ID | Serial no. | Variety name or collection region | Classification | Remarks |
|---|---|---|---|---|
| POP1 | 1-5 | Jihuang il ho | variety | High yielding |
| POP2 | 5-10 | Korea Jihuang | variety | Resistant to excessive humidity |
| POP3 | 11-15 | Yeoju, Korea* | accession | - |
| POP4 | 16-20 | Daekyeong | variety | High yielding |
| POP5 | 21-25 | Jinan, Korea* | accession | - |
| POP6 | 26-30 | Kumsan, Korea* | accession | - |
| POP7 | 31-35 | Uiseong, Korea* | accession | - |
| POP8 | 36-40 | Jeongup, Korea* | accession | - |
| POP9 | 41-45 | Gunwi, Korea* | accession | - |
| POP10 | 45-50 | Chuncheon, Korea* | accession | - |
| POP11 | 51-55 | Japan* | accession | High contents of catapol |

*: Collection region

from 36°C to 45°C, with one degree increase were tested with the PCR condition of 10 ng templet DNA, 4 μM primer, 1.0 unit *Taq* DNA Polymerase in a 25 μl total reaction volume. The optimum PCR condition obtained was as following: two minutes at 94°C for the initial denaturation; and 35 cycles of 30 seconds at 94°C, 30 seconds 42°C, 60 seconds 72°C for amplification reaction; and a final five minutes at 72°C for extension (data not shown).

Amplification products were analyzed by electrophoresis on 1.5% agarose gel in 1× TBE buffer and detected by ethidium bromide staining under UV lights. Only clear and distinct bands were scored both in agarose gels.

### Statistical analysis

All gel analysis were scored manually and independently, the presumed homologous RAPD bands were scored as present (1) or absent (0) to create a binary matrix, each of which was treated as an independent character regardless of its intensity. Genetic similarities were calculated based on the Dice's similarity coefficient described by Nei and Li (1979) as follows [10]: [2a/(2a+b+c)] where a is the number of bands common for genotype x and y, b is the number of bands present only in genotype x, and c is the number of bands present only in genotype y. First, based on genetic similarity matrix, dendrogram was constructed using the cluster analysis of the unweighted pair group method with arithmetic averages (UPGMA). Second, a principal coordinate analysis (PCA) was conducted, on the basis of similarity measures using the DCENTER and EIGEN procedures to cluster *R. glutinosa* accessions. All above statistical analysis were performed with NTSYS-pc (version 2.11a). Relationships of the *R. glutinosa* populations were estimated from the RAPD data using the UPGMA clustering method on the basis of Nei's (1978) unbiased genetic distance [9].

RAPD each individual allele was considered to be a single locus. The ability of the most informative primers to differentiate between isolates was assessed by calculating their Resolving power (Rp) [12]. Rp was calculated with the formula $Rp=\sum Ib$, where Ib (band informativeness) takes the value of 1-[2×(0.5-p)], and p is the proportion of 55 genotypes containing the band. The genetic variability was estimated to three ways: (i) the percentage of polymorphic loci (representing polymorphic bands), (ii) gene diversity (GD) and (iii) Shannon information index (I'- denotes the diversity of RAPD markers) [7]. Gene diversity of a RAPD each individual allele is calculated as $1-\sum Pi^2$, where Pi is the frequency of $i^{th}$ allele (band).

The occurrence of population structure was estimated by calculating total genetic diversity ($H_T$) of the polymorphic loci, mean genetic diversity for each RAPD marker within population ($H_S$) and genetic differentiation ($G_{ST}$) across populations as a proportion of total diversity [20].

## Results and Discussion

RAPD techniques have been applied to access the genetic diversity and relationships among and within three varieties and eight landraces in *R. glutinosa* at DNA level.

The percentage of polymorphic bands according to the application of selected primers was shown in Table 2. Ten

Table 2. The total number of scored bands ($S_B$), number of polymorphic bands ($P_B$), percentage of polymorphic loci (P %) and resolving power (Rp) of 10 RAPD markers in the 55 *R. glutinosa* samples

| Primer[a] | Sequence (5′ to 3′) | $S_B$ | $P_B$ | P (%) | $R_P$ |
|---|---|---|---|---|---|
| OPA-1 | CAGGCCCTTC | 2 | 1 | 50.0 | 0.145 |
| OPA-3 | AGTCAGCCAC | 11 | 8 | 72.7 | 2.945 |
| OPA-4 | AATCGGGCTG | 7 | 6 | 85.7 | 0.800 |
| OPA-5 | AGGGGTCTTG | 4 | 4 | 100.0 | 0.982 |
| OPA-9 | GGGTAACGCC | 9 | 8 | 88.9 | 2.909 |
| OPA-10 | GTGATCGCAG | 11 | 10 | 90.9 | 4.145 |
| OPA-11 | CAATCGCCGT | 13 | 13 | 100.0 | 5.091 |
| OPA-15 | TTCCGAACCC | 4 | 4 | 100.0 | 2.691 |
| OPA-17 | GACCGCTTGT | 5 | 5 | 100.0 | 1.527 |
| OPA-19 | CAAACGTCGG | 7 | 5 | 71.4 | 2.836 |
| Total | | 73 | 64 | 87.7 | 24.073 |

[a]Primers that reveal polymorphic fragments among and within varieties and accessions of *R. glutinosa*.

primers out of 20 primers, which produced polymorphic and reproducible bands, were selected for further analysis. The selected random primers were OPA-1, A-3, A-4, A-5, A-9, A-10, A-11, A-15, A-17 and A-19. All the chosen primers amplified fragments among and within the 11 populations studied, with the number of amplified fragments ranging two (OPA-1) to thirteen (OPA-11), which varied in size from 200 bp to 1,400 bp. Among the 73 amplified DNA bands, 64 were polymorphic, with an average of 6.4 polymorphic fragments per primer. Percentage polymorphism ranged from a minimum of 50% (OPA-1) to a maximum of 100% (OPA-11), with an average of 87.7% polymorphism. Six out of selected ten primers showed more than 60% polymorphism. The Resolving power (Rp) values varied from 0.145 for the OPA-1 primer to 5.091 for the OPA-11 primer with collective rate of 24.073 (Table 2).

Fig. 1 is the representative of the extent of polymorphism observed among 11 populations as revealed by OPA-11 and OPA-19 primers. Especially, OPA-10, OPA-11 and OPA-19 primers showed specific bands for the breeding line of Korea Jiwhang, which could be useful for discriminating from other varieties and landraces of *R. glutinosa*. The molecular sizes of specific bands were 1,200 bp, 1,400 bp and 900 bp, respectively. Also, the result generated by OPA-10 and OPA-11 primers showed the specific band for the cultivar of Jiwhang il ho at 1,300 bp. Therefore, RAPD markers derived from this study will be useful tool for differentiating varieties of *R. glutinosa* from other varieties and landraces of *R. glutinosa* at DNA level.

A.

B.

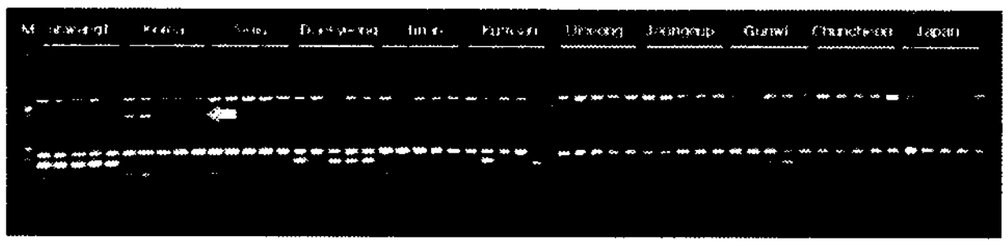

Fig. 1. Profiles of PCR products obtained from genomic DNA using the 10-based OPERON primers, OPA-11 (A) and OPA-19 (B). Lane M, 100 bp DNA ladder; Lane 1-5, Jiwhang il ho; Lane 5-10, Korea Jiwhang; Lane 11-15, Yeoju accession; Lane 16-20, Daekyeong Jiwhang; Lane 21-25, Jinan accession; Lane 26-30, Kumsan accession; Lane 31-35, Uiseong accession; Lane 36-40, Jeongup accession; Lane 41-45, Gunwi accession; Lane 46-50, Chuncheon accession; Lane 51-55, Japanese accession.

There were limited number of studies conducted with molecular markers in *R. glutinosa* compared with other major crops and medicinal herbs. Zhou et al. (2004) reported that RAPD and ISSR markers were suitable for germplasm assessment of genetic diversity in *R. glutinosa*, and ISSR maker was superior to RAPD maker [22]. To provide molecular evidence for its breeding by studying the genetic relationship among varieties of *R. glutinosa* in China, eight varieties of group Beijing have a close genetic relationship which provides information for *R. glutinosa*'s breeding [3].

A total of 73 reproducible band types was scored and analyzed for computation of similarity matrix and genetic distance/relatedness. Genetic distances were evaluated ranging from 0.586 between Jiwhang il ho and Korea Jiwhang to 0.978 between Uiseong accession and Chuncheon accession (Table 3). The smallest genetic distance was observed between Uiseong landrace and Chunchon landrace and the largest between Jiwhang il ho and Korea Jiwhang. Genetic distance values were used to construct a dendrogram. Cluster analysis of RAPD markers showed that populations of *R. glutinosa* were classified into four different groups. It was also shown that two populations, such as Korea Jiwhang and Jiwhang il ho, were clearly separated by two-dimensional plot resulted in analyzing genetic diversity of the 55 accessions in *R. glutinosa*. Jiwhang il ho and Korea Jiwhang were promising lines improved by selection-breeding at National Institute of Crop Science in Korea. Since uniformities of those lines are higher than other landraces, they were classified into each one group by cluster analysis and principal component analysis (Fig. 2 and Fig. 3).

Table 3. Genetic distances determined by 10 RAPD primers in 11 populations

| POP ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 0.414 | | | | | | | | | |
| 3 | 0.169 | 0.221 | | | | | | | | |
| 4 | 0.118 | 0.394 | 0.105 | | | | | | | |
| 5 | 0.294 | 0.142 | 0.098 | 0.191 | | | | | | |
| 6 | 0.153 | 0.289 | 0.052 | 0.074 | 0.110 | | | | | |
| 7 | 0.194 | 0.288 | 0.086 | 0.067 | 0.107 | 0.096 | | | | |
| 8 | 0.222 | 0.302 | 0.076 | 0.134 | 0.126 | 0.076 | 0.050 | | | |
| 9 | 0.250 | 0.128 | 0.118 | 0.155 | 0.108 | 0.110 | 0.067 | 0.088 | | |
| 10 | 0.166 | 0.325 | 0.080 | 0.069 | 0.129 | 0.085 | 0.022 | 0.045 | 0.085 | |
| 11 | 0.266 | 0.228 | 0.116 | 0.157 | 0.074 | 0.116 | 0.075 | 0.082 | 0.094 | 0.071 |

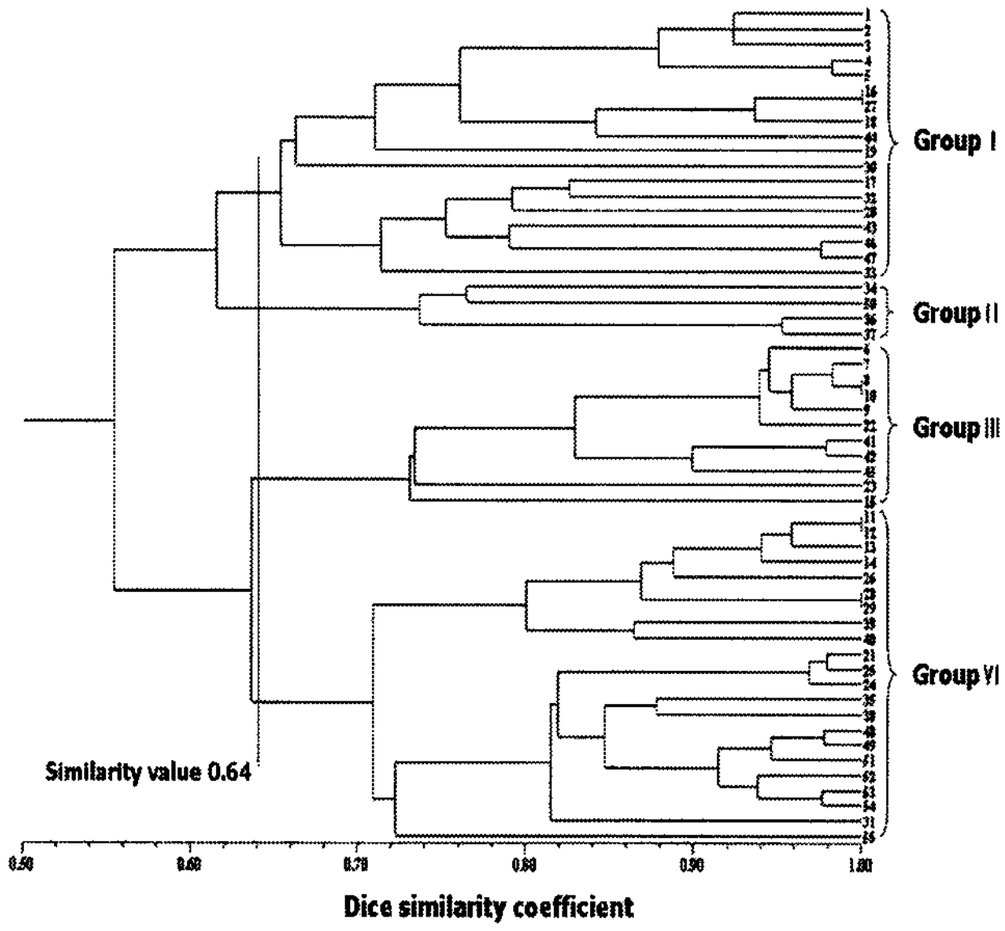


Fig. 2. Dendrogram based on Dice similarity coefficient by UPGMA using 73 RAPD bands of 11 genotypes in *R. glutinosa*. Population numbers are same as Table 1.

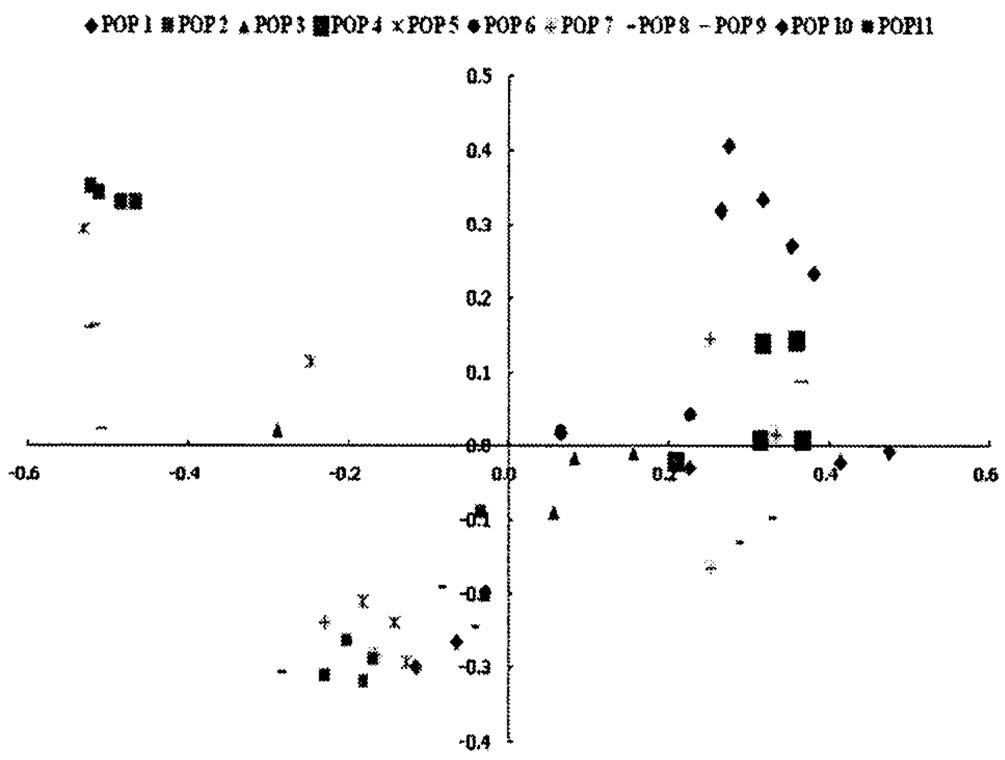


Fig. 3. Two-dimensional plot of genetic diversities of the 55 accessions *in R. glutinosa* revealed by the PCA based on 10 RAPD markers.

Average of genetic diversity within the population ($H_S$) was 0.110, while average of total genetic diversity ($H_T$) was 0.229. Across all RAPD makers the $G_{ST}$ value was 0.517 (Table 4), indicating that about 52% of the total genetic variation could be explained by RAPDs differences while the remaining 48% might be attributable to differences among samples. These results indicate that genetic differentiation was relatively high in *R. glutinosa* populations and genetic diversity was low within the total sample and individual populations. Genotypic diversity varied among plant origins, based on Shannon indices. The diversity within regions ranged from 0.108 in Japan to 0.341 in Korea populations. Compared to gene diversity ($H_T$) the higher genotypic diversity (I) observed suggested that genotypic diversity is distributed mainly among population lineage (Table 4).

Table 4. Estimates of genetic diversity in *R. glutinosa* using 10 RAPD markers

| Comparison level | P | I | $H_T$ | $H_S$ | $G_{ST}$ |
|---|---|---|---|---|---|
| Population | 87.7 | 0.355 (0.247) | 0.229 (0.033) | 0.11 (0.008) | 0.517 (0) |
| Classification[a] | | | | | |
| Variety | 67.1 | 0.348 (0.283) | 0.233 (0.041) | 0.067 (0.007) | 0.714 (0) |
| accession | 74.0 | 0.322 (0.261) | 0.208 (0.035) | 0.135 (0.014) | 0.352 (0) |
| Origin | | | | | |
| Korea | 86.3 | 0.341 (0.25) | 0.219 (0.033) | 0.101 (0.007) | 0.542 (0) |
| Japan | 20.6 | 0.108 (0.227) | 0.073 (0.025) | 0.073 (0.025) | 0 (0) |

[a]: except Japan population
P: percentage of polymorphic *loci*
I: shannon information index
$H_T$: total genetic diversity
$H_S$: genetic diversity within population
$G_{ST}$: the proportion of total genetic variation due to differences among populations
( ): standard deviation

In conclusion, the RAPD technique will be used for useful method to discriminate superior varieties from other varieties and landraces in *R. glutinosa* and for the application of marker-assisted selection tools to genetic enhancement of cultivated Jihuang for desirable traits.

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## 초록 : 분자표지자에 의한 지황 유전집단의 유전적 다양성

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RAPD 분석을 이용하여 지황 육성 계통과 지역 수집종 들을 구분할 수 있는 분자표지자를 선발하고, 집단 간, 집단 내 유전적 다양성을 평가하기 위하여 본 실험을 수행하였다. 총 20개의 임의 primer를 이용하여 PCR 한 결과, 육성 계통과 수집종 들을 구별할 수 있는 OPA-1 등 10개의 재현성과 다형성이 좋은 프라이머 들을 선발하였다. 특히 OPA-10, OPA-11 및 OPA-19는 고려지황과 지황1호를 다른 계통 및 수집종 들과 구별할 수 있었으며, 이들 프라이머를 이용하여 0.9 kb, 1.2 kb, 1.3 kb 및 1.4 kb 등의 육성계통 특이적인 DNA 밴드들을 확보할 수 있었다. 한편 이들의 결과를 토대로 통계처리에 의한 유전분석 결과, 고려지황, 지황1호 및 일본지황은 집단 내 유사도가 높아 다른 집단들과 구별되었다. 결론적으로, RAPD 분석을 통한 결과는 지황의 유전적 다양성 이해와 특정 계통을 다른 계통 및 수집종 들과 구분할 수 있는 방법으로 이용될 수 있다.